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(57) Abstract

The present invention provides peptides of general formula (I): X-Ala-Arg-Arg-J wherein X is H or one or more amino acids and J is OH or one or more amino acids, and pharmaceutically acceptable derivatives thereof, which peptides contain 3 to 15 amino acid residues. The invention also provides antisense oligonucleotides and derivatives thereof corresponding to the DNA coding for ζ-PKC, especially the oligonucleotide having the sequence GGTCCTGCTGGGCAT (SEQ ID NO 14). The novel peptides and oligonucleotides are of use in medicine for the treatment of conditions whose underlying aetiology is associated with ζ-PKC activity, for example tumours, hyperproliferative disorders and viral infections. Processes for preparing the novel compounds, pharmaceutical compositions containing them and their use in medicine are described.

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INHIBITOR OF PROTEIN KINASE C

This invention relates to a novel method of treatment and in particular to novel peptide inhibitors of a specific protein kinase C isotype, to processes for their preparation, to pharmaceutical compositions containing them and to their use in medicine.

Considerable effort has been invested to identify critical steps in mitogenic signal transduction pathways. Phospholipid degradation, which is potently activated following stimulation with growth factors (M J Berridge, Annu. Rev. Biochem. 56, 159 (1987) and J H Exton, J. Biol. Chem. 265, 1 (1990)) is the core of recent intense research. Although most of the work has been focused on phosphoinositide (PI) turnover, a number of studies demonstrate the existence of PI-independent signal transduction cascades involving the phosphodiesterase-mediated hydrolysis of phosphatidylcholine (PC) (J H Exton (1990, loc cit); J M Besterman et al, Proc. Natl. Acad. Sci. USA 83, 6785 (1986); Lacal et al., Nature 330, 269 (1987a); M S Pessin et al, J. Biol. Chem. 265, 7959 (1990) and P Larrodera et al., Cell 61, 1113 (1990)). Recently, evidence has accumulated showing that activation of phospholipase C catalyzed hydrolysis of phosphatidylcholine (PC-PLC) is sufficient to mimic a significant portion of the platelet-derived growth factor (PDGF) mitogenic signal (P. Larrodera et al. (1990), loc cit.). PLC-mediated PC hydrolysis has also been shown to be stimulated by the product of the ras oncogene, ras p21 (J C Lacal et al. (1987a) loc cit; B D Price et al, J. Biol. Chem. 264, 16638 (1989); I Diaz-Laviada et al., EMBO J 9, 3907 (1990); M Lopez-Barahona et al., J. Biol. Chem. 265, 9022 (1990)) whose role in mitogenic cascades has been demonstrated (M R Smith et al., Nature 320, 540 (1986).

Oocytes from Xenopus laevis are a suitable system for investigating the involvement of different enzymatic activities in relevant signal transduction mechanisms controlled by oncogenes (L J Korn et al., Science 236, 840 (1987); J C Lacal et al., Science 238, 533 (1987b)). Thus, Xenopus oocytes undergo a maturation program following stimulation with either insulin or progesterone, and several lines of evidence indicate the specific involvement of ras p21 in the maturation signalling cascades activated by insulin/IGF- 1:

1. microinjection of ras p21 activates maturation in oocytes (C. Birchmeier et al., Cell 43, 615 (1985)); and
2. microinjection of a neutralizing anti-ras p21 antibody (Y13- 259) blocks the maturation program induced by insulin but not by progesterone (L J Korn et al., (1987) loc cit.).

A more definitive proof of the involvement and importance of PC-PLC in the oocyte maturation pathway activated by insulin/ras p21 has recently been obtained (Garcia de Herreros et al., J. Biol. Chem. 266, 6825-6829 (1991)). Thus, it has been shown that PLC-mediated hydrolysis of PC is both necessary and sufficient for activation of maturation in Xenopus laevis oocytes by insulin/ras p21, as measured by induction of germinal vesicle breakdown (GVBD) and activation of maturation promoting factor H1 kinase. All these results permit one to suggest that PC-PLC activation could be critically involved in pathways controlling cell growth and tumour transformation.

Although the biochemical parameters controlling oocyte maturation have been shown to be identical to those involved in mitogenesis in mammals (A.W. Murray & M. W. Kirschner, Nature 339, 275 (1989), other model systems useful for this type of study include cultures of somatic mammalian cells, particularly mouse fibroblasts stimulated by specific growth factors or serum.

Nevertheless, the mechanism whereby PC-PLC transduces growth factor mitogenic signals remains to be elucidated. Since PLC-mediated PC hydrolysis generates diacylglycerol (DAG), which is an important activator of protein kinase C (PKC) (Y. Nishizuka, Nature 334, 661 (1988)), the involvement of this kinase in the mitogenic signalling cascades activated by PC-PLC is an intriguing possibility.

Two major categories of PKC's have been cloned and characterised to date with multiple subspecies within these two categories (see Y. Nishizuka, Science 258, 607-614 (1992)for review). These isotypes all contain a conserved pseudosubstrate autoinhibitory domain (T.S. Soderling, J.Biol.Chem. 265, 1823 (1990)). A synthetic peptide corresponding to residues 19 to 31 of the pseudosubstrate region of α, β and γ isotypes of PKC has been shown to be a potent inhibitor of rat brain PKC activity in an in vitro assay system (C. House & B. E. Kemp, Science 238, 1726 (1987)).

We have now found certain peptides which inhibit the activity of protein kinase C isotype zeta (ζ).

Accordingly, the present invention provides peptides of general formula (I)

X-Ala-Arg-Arg-J (I)

wherein X is H or one or more amino acids and J is OH or one or more amino acids, and pharmaceutically acceptable derivatives thereof, which peptides contain 3 to 15 amino acid residues.

As used herein the term "derivatives" includes salts and solvates of the peptides according to the invention and peptides according to the invention which contain one or more end group modified structures, with the proviso that the C-terminal amino acid residue is other than an alkyl ester of arginine. End group modified structures include deletion, acylation (for example acetylation or benzoylation), alkylation, fatty acylation (for example myristoylation) or cyclisation of the N-terminal amine group and deletion, amidation (including mono- and dialkylamidation), cyclisation or reduction of the C-terminal carboxy group. N-acyl, for example N-acetyl derivatives are preferred.

Suitable salts of the peptides of formula (I) include physiologically acceptable acid addition salts derived from inorganic and organic acids, such as hydrochlorides, hydrobromides, sulphates, nitrates, oxalates, phosphates, tartrates, acetates, citrates, trifluoroacetates, fumarates, maleates, succinates and sulphonates e.g. p-toluenesulphonates and methanesulphonates. Suitable solvates of the peptides of formula (I) include, for example, hydrates.

The peptides according to the invention are specific inhibitors of protein kinase C isotype zeta (ζ–PKC). As used herein, the term "protein kinase C isotype zeta" (ζ–PKC) means any subspecies of protein kinase C which contains the specific autoinhibitory pseudosubstrate domain RRGARRWRK (SEQ ID NO. 5). This specific sequence has been found to be perfectly (100%) conserved in ζ–PKC variants isolated from a number of different sources including rat brain ζ–PKC (Ono et al., Proc.Natl.Acad.Sci.USA, 86, 3099-3103 (1989)) and Xenopus laevis ζ–PKC and ζn-PKC (present work).

Preferred peptides of general formula (I) contain 3 to 9 amino acid residues, particularly 3 to 6 amino acid residues.

In a preferred group of peptides of general formula (I) X is H, acetyl, Gly, Arg-Gly or Arg-Arg-Gly and J is OH, Trp, Trp-Arg or Trp-Arg-Lys.

Preferred peptides according to the invention include :

Ala-Arg-Arg (SEQ ID NO. 1);
Ala-Arg-Arg-Trp (SEQ ID NO. 2);
Ala-Arg-Arg-Trp-Arg (SEQ ID NO. 3);
Ala-Arg-Arg-Trp-Arg-Lys (SEQ ID NO. 4);
Arg-Arg-Gly-Ala-Arg-Arg-Trp-Arg-Lys (SEQ ID NO. 5); and
N-acetyl-Ala-Arg-Arg (SEQ ID NO. 17);
and pharmaceutically acceptable derivatives thereof.

It will be appreciated that the peptides of formula (I) contain at least three chiral centres. It is to be understood that formula (I) is intended to encompass all diastereoisomers of the peptides of the invention as well as mixtures thereof, including racemates. The amino acids may be either of the natural L-form or of the D-form or a mixture e.g. a racemic mixture, of L- and D-forms. Nevertheless, the peptides of formula (I) preferably contain the natural L-forms of the amino acid residues contained therein.

The peptides according to the invention are prepared in substantially pure form and are substantially free of other peptides or amino acids. Preferably the peptides have a purity of 90% or higher, for example 95%, although a purity of 98% or higher (based on all peptides present) is preferred for clinical use.

The novel peptides of this invention can be made by appropriate adaptation of conventional methods for peptide synthesis. Thus, the peptide chain can be prepared by a series of coupling reactions in which the constituent amino acids are added to the growing peptide chain in the desired sequence. The use of various N-protecting groups, e.g. the carbobenzyloxy group or the t-butyloxycarbonyl group (BOC), various coupling reagents, e.g. dicyclohexylcarbodiimide or carbonyldimidazole, various active esters, e.g. esters of N-hydroxypthalimide or N-hydroxysuccinimide, and various cleavage reagents, e.g. trifluoroacetic acid, HCl in dioxane, boron tris-(trifluoroacetate) and cyanogen bromide, and reaction in solution with isolation and purification of intermediates is well-known classical peptide methodology.

Preferably, the peptides of this invention are prepared by the well-known Merrifield solid support method (Merrifield, J. Amer. Chem. Soc. 85, 2149-54 (1963) and Science 150, 178-85 (1965)).

Thus, a further aspect of the invention provides a process for preparing a peptide as described herein which comprises

(a) binding the desired protected carboxy-terminal amino acid to a suitable solid support;

(b) reacting other protected amino acids with the support-bound carboxy-terminal amino acid in the desired sequence; and

(c) removing the protecting groups and releasing the peptides so-obtained from the solid support.

This procedure, though using many of the same chemical reactions and blocking groups of classical peptide synthesis, provides a growing peptide chain anchored by its carboxyl terminus to a solid support, usually cross-linked polystyrene or styrene divinylbenzene copolymer. This method conveniently simplifies the number of procedural manipulations since removal of the excess reagents at each step is effected simply by washing of the solid support.

Thus, the carboxyl terminal amino acid, having its alpha-amino group suitably protected, is coupled to a solid support. After removal of the alpha-amino protecting group, for example by using trifluoroacetic acid in methylene chloride, the next step in the synthesis is ready to proceed. Other standard cleaving reagents and conditions for the removal of specific amino protecting groups may be used, as described in the literature.

The remaining alpha-amino- and side-chain-protected amino acids are then coupled stepwise in the desired order to obtain an intermediate peptide connected to the solid support. As an alternative to adding each amino acid separately in the synthesis, some of them may be coupled to one another prior to the addition to the growing solid-phase chain. The selection of the appropriate coupling reagents is within the skill of the art.

Common to chemical syntheses of peptides is the protection of the labile side-chain groups of the various amino acid moieties with suitable protecting groups at that site until the group is ultimately removed after the chain has been completely assembled. Also common is the protection of the alpha-amino group on an amino acid whilst that entity reacts at the carboxyl group followed by the selective removal of the alpha-amino-protecting group to allow subsequent reaction to take place at that location. Accordingly, it is common that, as a step in the synthesis, an intermediate compound is produced which includes each of the amino acid residues located in the desired sequence in the peptide chain with various of these residues having side-chain protecting groups. These

protecting groups are then commonly removed substantially at the same time so as to produce the desired resultant product following purification.

After the desired amino-acid sequence has been completed, the intermediate peptide is removed from the solid support by treatment with a reagent, such as liquid HF, which not only cleaves the peptide from the support but also cleaves all the remaining side-chain protecting groups. The peptide can then be purified for example by gel permeation, HPLC (Rivier et al., Peptides : Structure and Biological Function (1979) pp. 125-128), ion exchange gel and partition chromatography, countercurrent distribution or other known methods.

Where it is desired to isolate a peptide of the invention as a salt, for example as an acid addition salt, this may be achieved by treating the free base of the peptide with an appropriate acid, preferably with an equivalent amount of acid.

The peptides according to the invention have been found to be potent and specific inhibitors of ζ–PKC. Accordingly the peptides are useful as scientific tools for studying the role of ζ–PKC within biological systems, particularly its role in mitogenic signalling pathways, for example in the maturation of germ cells and the proliferation of somatic mammalian cells.

Thus, the present invention provides a method for inhibiting cellular ζ–PKC activity which comprises contacting cells capable of responding to ζ–PKC activation with an effective amount of an inhibitor of ζ–PKC. Whether a given compound is an inhibitor of ζ–PKC and therefore encompassed by this invention may be readily determined by routine experimentation, for example using an in vitro assay system as previously described for example by Ono et al 1989, loc.cit., or as described below. Useful ζ–PKC inhibitors preferably exhibit the potency and selectivity demonstrated herein for the preferred peptide according to the invention Ala-Arg-Arg (SEQ.ID.NO.1). Suitable assay systems include, for example, phosphorylation assays using exogenous ζ–PKC isolated from a suitable tissue source, for example, rat brain, bovine brain or X.laevis oocytes, or using a source of recombinant ζ–PKC, such as that described by Ono et al (1989) loc.cit. Alternative assay protocols may employ appropriate microinjection techniques such as those described herein, for example analysis of oocyte maturation pathways such as H1-kinase and germinal vesicle breakdown, or analysis of mammalian cell proliferation, for example by assaying

DNA synthesis. Such methods are useful for in vitro studies of ζ–PKC activity, particularly its role in mitogenic signalling pathways, and as screening methods for the selection of novel ζ–PKC inhibitory agents. Suitable in vivo screens may be established by conventional techiques employing transgenic animal e.g. mouse models.

A further aspect of the invention therefore provides a screening method for the selection of agents suitable for the prevention or treatment of pathological conditions mediated by ζ–PKC activity which comprises

a) incubating a sample comprising the agent to be tested with an assay system capable of indicating inhibition and/or activation of ζ–PKC activity;

b) determining whether and, if desired, the extent to which ζ–PKC activity is altered by said agent; and

c) selecting agents determined to be potent and selective ζ–PKC inhibitors.

The applicants have found that ζ–PKC is a critical step in the transduction of mitogenic signals in response to the insulin/ras p21/PC-PLC specific pathway. Preliminary evidence also suggests that ζ–PKC may play a role in the cascade of events linking cell activation to viral genome transcription. The in vivo significance of ζ–PKC had not hitherto been appreciated and thus the present applicants are the first to recognise that in vitro inhibitors of ζ–PKC may have in vivo utility in medicine for the diagnosis, prevention or treatment of pathological conditions mediated by ζ–PKC activity. In particular the applicants have demonstrated in appropriate models that ζ–PKC inhibitors may be effective against a wide spectrum of tumours, hyperproliferative disorders such as psoriasis, and viral infections such as HIV.

As used herein, the term "tumour" means both benign and malignant tumours, cancers, cancerous growths or neoplasms. These tumours include, but are not limited to, a wide spectrum of mammalian (including human) tumours such as carcinomas, adenocarcinomas, melanomas, sarcomas, lymphomas and leukemias. Specific examples include cancers of the oral cavity and pharynx (lip, tongue, mouth, pharynx), oesophagus, stomach, small intestine, large intestine, rectum, liver and biliary passages, pancreas, larynx, lung, bone, connective tissue, skin, colon, breast, cervix uteri, corpus endometrium, ovary, prostate, testis, bladder, kidney and other urinary tissues, eye, brain and central nervous system, thyroid and other endocrine glands, leukemias (lymphocytic, granulocytic, monocytic), Hodgkin's disease, non-Hodgkin's lymphomas,

multiple myeloma etc. The applicants have shown that specific tumours, for example maxilofacial squamous cell carcinoma, are associated with unusually high levels of PKC isotype ζ. Furthermore, overexpression of ζ–PKC, by using a plasmid with a potent viral promoter in NIH 3T3 fibroblasts confers to these cells severe deregulation of their growth properties including a reduced serum dependence, lower doubling times and higher saturation densities, as well as colony formation in semi-solid medium.

Accordingly the peptides of formula (I) are effective against a wide spectrum of tumours, hyperproliferative disorders and viral infections in mammals, including humans, and may be used for the treatment of cancer, hyperproliferative disorders such as psoriasis, and viral infections such as HIV.

The invention thus further provides peptides of formula (I) and their physiologically acceptable derivatives for use as active therapeutic agents, in particular for the treatment of conditions whose underlying aetiology is associated with ζ–PKC activity in animals (especially humans).

In a particular aspect of the present invention there is provided a peptide of formula (I) or a physiologically acceptable derivative thereof for use in the treatment of cancer, for example maxilofacial squamous cell carcinoma.

In a further or alternative aspect there is provided a method for inhibiting the activity of ζ–PKC in a mammal including a human comprising administration of an effective amount of a peptide of formula (I) or a physiologically acceptable derivative thereof.

There is also provided in a further or alternative aspect use of a peptide of formula (I) or a physiologically acceptable derivative thereof for the manufacture of a medicament for inhibiting ζ–PKC activity in a mammal.

It will be appreciated by those skilled in the art that reference herein to treatment extends to prophylaxis as well as the treatment of established symptoms.

It is possible that a peptide of the invention may be administered to a patient as the raw chemical, but it is preferable to present the active ingredient as a pharmaceutical formulation.

The invention accordingly provides a pharmaceutical formulation comprising a peptide of formula (I) or a physiologically acceptable derivative thereof together with one or more physiologically acceptable carriers and, optionally, other therapeutic and/or prophylactic ingredients. The carriers must be

"acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof.

Pharmaceutical formulations include those suitable for oral, rectal, nasal, topical, implant or parenteral (including intramuscular, subcutaneous and intravenous) administration or in a form suitable for administration by inhalation or insufflation. The formulations may, where appropriate, be conveniently presented in discrete dosage units and may be prepared by any of the methods well known in the art of pharmacy. All methods include the step of bringing into association the active compound with liquid carriers or finely divided solid carriers or both and then, if necessary, shaping the product into the desired formulation.

For oral administration, the pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g. pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (e.g. lactose, microcrystalline cellulose or calcium phosphate); lubricants (e.g. magnesium stearate, talc or silica); disintegrants (e.g. potato starch or sodium starch glycollate); or wetting agents (e.g. sodium lauryl sulphate). The tablets may be coated by methods well known in the art. Liquid preparations for oral administration may take the form of, for example, solutions, syrups or suspensions, or they may be presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g. sorbitol syrup, methyl cellulose or hydrogenated edible fats); emulsifying agents (e.g. lecithin or acacia); non-aqueous vehicles (e.g. almond oil, oily esters or ethyl alcohol); and preservatives (e.g. methyl or propyl-p-hydroxybenzoates or sorbic acid).

For topical administration in the mouth, the pharmaceutical compositions may take the form of buccal or sub-lingual tablets, drops or lozenges formulated in conventional manner.

For topical administration to the epidermis the peptides may be formulated as creams, gels, ointments or lotions or as a transdermal patch. Such compositions may for example be formulated with an aqueous or oily base with the addition of suitable thickening, gelling, emulsifying, stabilising, dispersing, suspending, and/or colouring agents.

The peptides of the invention may also be formulated as depot preparations. Such long acting formulations may be administered by implantation (for example subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the peptides may be formulated with suitable polymeric or hydrophobic materials (for example as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example as a sparingly soluble salt.

The peptides of the invention may be formulated for parenteral administration by injection, conveniently intravenous, intramuscular or subcutaneous injection, for example by bolus injection or continuous intravenous infusion. Formulations for injection may be presented in unit dosage form e.g. in ampoules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilising and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g. sterile pyrogen-free water, before use.

The peptides of the invention may also be formulated in rectal compositions such as suppositories or retention enemas, e.g. containing conventional suppository bases such as cocoa butter or other glyceride.

For intranasal administration the peptides of the invention may be used, for example, as a liquid spray, as a powder or in the form of drops.

For administration by inhalation the peptides according to the invention are conveniently delivered in the form of an aerosol spray presentation from pressurised packs or a nebuliser, with the use of a suitable propellant, e.g. dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane, carbon dioxide or other suitable gas. In the case of a pressurised aerosol the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of e.g. gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of a peptide of the invention and a suitable powder base such as lactose or starch.

Any of the pharmaceutical compositions described above may be presented in a conventional manner associated with controlled release forms.

Preferably the pharmaceutical compositions according to the invention are suitable for intranasal, topical or parenteral administration.

It will be appreciated that the amount of a peptide of formula (I) required for use in treatment will vary not only with the particular peptide selected, but also with the route of administration, the nature of the condition being treated and the age, weight and condition of the patient and will ultimately be at the discretion of the attendant physician or veterinarian. In general, however, a suitable dose will be in the range of from about 1 to about 500mg per day, preferably in the range of 20 to 200mg per day, most preferably in the range of 50 to 120mg per day.

A suitable daily dose for use in prophylaxis will generally be in the range of 0.1 mg to 50mg.

The desired dose may conveniently be presented in a single dose or as divided doses administered at appropriate intervals, for example as two, three, four or more sub-doses per day. The peptide is conveniently administered in unit dosage form. A convenient unit dosage formulation contains the active ingredient in an amount of from 0.1 to about 500mg.

The peptides of the present invention may also be used in combination with other therapeutic agents, for example, other anticancer agents. In particular the compounds of the invention may be employed together with known anticancer agents.

The invention thus provides, in a further aspect, a combination comprising a peptide of formula (I) as defined herein together with another therapeutically active agent, in particular an anticancer agent.

The combination referred to above may conveniently be presented for use in the form of a pharmaceutical formulation and thus pharmaceutical formulations comprising a combination as defined above together with a pharmaceutically acceptable carrier therefor comprise a further aspect of the invention.

When peptides of formula (I) are used in combination with a second therapeutic agent, the active compounds may be administered either sequentially or simultaneously by any of the routes described above.

Suitable therapeutic agents for use in the combinations defined above include, for example alkylating agents such as cyclophosphamide, antimetabolites such as methotrexate, mitotic inhibitors such as vinblastine, antitumour antibiotics such as adriamycin, endocrine therapy such as tamoxifen,

flutamide, goserelin acetate and medroxyprogesterone acetate, radiotherapy and immunotherapy.

When peptides of formula (I) are used in combination with a second therapeutic agent the dose of each active compound may vary from that when the compound is used alone. Thus when peptides of formula (I) are used together with a second therapeutic agent the dose of each active compound may be the same or different to that employed when the compound is used alone. Appropriate doses will be readily appreciated by those skilled in the art.

In a further aspect the present invention provides antisense oligonucleotides and derivatives thereof corresponding to the DNA coding for ζ–PKC, preferably the regulatory domain of ζ–PKC DNA, or a degenerate equivalent thereof. Preferred antisense oligonucleotides are fifteen-mer antisense oligonucleotides corresponding to the beginning of the coding region of ζ–PKC, preferably of mammalian ζ–PKC, especially human ζ–PKC. An example of a suitable antisense oligonucleotide according to the invention is:

GGTCCTGCTGGGCAT (SEQ ID NO 14)

or a derivative or a degenerate equivalent thereof.

The antisense oligonucleotides are preferably modified on the backbone to phosphorothioates to reduce nuclease degradation.

The antisense oligonucleotides and equivalents thereof according to the invention may be prepared in conventional manner as described hereinafter.

The antisense oligonucleotides according to the present invention have been found to be potent and specific inhibitors of ζ–PKC and have utility both as in vitro scientific tools and in vivo for the treatment of pathological conditions mediated by ζ–PKC activity, in particular tumours, hyperproliferative disorders such as psoriasis, and viral infections such as HIV. Suitable pharmaceutical compositions containing the antisense oligonucleotides according to the invention and dosages thereof are as described for the peptides according to the invention as hereinbefore described.

The invention is further illustrated by the following non-limiting examples and accompanying figures. The common three-letter abbreviations or one-letter symbols for natural forms of amino acids and the standard one-letter codes for nucleotide residues are used throughout.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1

The involvement of ζ–protein kinase C in the activation of H1-kinase.

Stage VI oocytes either untreated or microinjected with 1ng of purified bovine brain PKC (Nishizuka (1988), Science 334, 61) were microinjected with either buffer control (speckled bars) or with 5μM (final concentration into the oocyte) of a mixture of the three peptides corresponding to the pseudosubstrate region of PKC isotypes α, β, and γ (Peptide A, SEQ ID NO. 6), δ (Peptide D, SEQ ID NO. 7) and ε (Peptide E, SEQ ID NO. 8) (striped bars) or with the peptide corresponding to the pseudosubstrate region of ζPKC (Peptide Z, SEQ ID NO. 5) (1μM) (black bars). Afterwards, oocytes were microinjected with 25μU of B. cereus PC-PLC or with 20ng of transforming v-H-ras p21, or they were incubated in the presence of PMA (100ng/ml), insulin (1μM) or progesterone (1μM). The reactions were stopped by 2h after stimulation, and H1-kinase activity was measured following precipitation of extracts with $p13^{suc1}$ linked to agarose beads. The pseudosubstrate peptides have the following sequences :
(A) peptide specific for PKC isotypes α, β, γ : RKGALRQKN (SEQ ID NO. 6);
(D) peptide specific for PKC δ : RRGAIKQAK (SEQ ID NO. 7);
(E) peptide specific for PKC ε : RQGAVRRRV (SEQ ID NO. 8);
(Z) peptide specific for PKC ζ : RRGARRWRK (SEQ ID NO. 5).

The control level of H1-kinase was 76 fmol/min/oocyte and was not affected by microinjection of pseudosubstrates. Essentially identical results were obtained in three other experiments.

Figure 2.

Immunoblot analyses of different PKC isotypes in extracts from Xenopus oocytes.

Oocyte extracts were resolved by SDS-PAGE, electroblotted and incubated with antibodies specific for PKC isotypes α, β and γ (A) or ζ (B), either in the absence or in the presence of the corresponding isoenzyme-specific peptides. Essentially identical results were obtained in three independent experiments.

Figure 3.

Effect of peptides A (SEQ ID NO. 6) and Z (SEQ ID NO. 5) on the autophosphorylation activity of ζ–PKC.

Oocyte extracts were incubated with 10μg of anti-ζ–PKC antibody and immunocomplexes were recovered with protein G-agarose. Immunoprecipitates were assayed for the autophosphorylation kinase activity of ζ–PKC in a mixture of 300μM [γ-^{32}P]ATP with or without 50μg/ml phosphatidylserine (PS). Incubations were carried out with different concentrations of peptides A or Z (described in the legend to Figure 1). Reactions were stopped by 45 min and proteins resolved by SDS-PAGE. No kinase activity was detected when oocyte extracts were incubated with protein G-agarose in the absence of antibody. Essentially identical results were obtained in three independent experiments.

Figure 4.

Immunoblot analyses of ζ–PKC levels in extracts from RNA-microinjected oocytes.

Sense and antisense RNAs synthesized in vitro from a plasmid harboring a fragment from the regulatory domain of ζ–PKC were microinjected into stage VI oocytes. Forty-eight hr following microinjection of RNAs or distilled water, oocytes were homogenized and extracts were resolved by SDS-PAGE, electroblotted and incubated with antibodies specific for PKC isotypes α, β and γ (A) or ζ (B). Essentially identical results were obtained in three independent experiments.

Figure 5.

Involvement of ζ–PKC in the activation of H1 kinase in Xenopus oocytes.

(A) Stage VI oocytes were microinjected with water (empty bars) or with 25 ng of either sense (striped bars) or antisense (black bars) synthetic RNAs. (B) Another set of oocytes were microinjected with water (empty bars) or with 150 ng per oocyte of either sense (striped bars), antisense (black bars) or nonsense (speckled bars) oligonucleotides specific for ζ–PKC. Subsequently, oocytes were microinjected with 20ng of transforming v-H-ras p21 or with 25 μU of B.cereus PC-PLC, or incubated in the presence of insulin (1μM) or progesterone (1μM). Afterwards, H1 kinase activity was determined in the extracts as described under Experimental Procedures, when oocytes displayed

a 50% induction of GVBD. Results are mean ± SD of three independent experiments with incubations in duplicate.

Figure 6

A comparison of sequences corresponding to the different pseudosubstrates.

Figure 7

The effect of different deletions of peptide Z (SEQ ID NO.5) on the activation of H1-kinase.

Stage VI oocytes, prepared following standard procedures, were microinjected with either buffer control or with 1 μM (final concentration into the oocyte) of peptides Z, Z1, Z2, Z3, Z4 or A4 (SEQ ID NOS. 5, 4, 3, 2, 1 or 9 respectively). Afterwards, oocytes were either microinjected with 25μU of B. cereus PC-PLC, or 20ng of ras p21, or they were incubated with insulin (1μM) or progesterone (1μM). Subsequently, reactions were stopped by 2h after stimulation, and H1-kinase activity was measured as described in the legend to Figure 1. The control level of H1-kinase was 80 fmol/min/oocyte and was not affected by microinjection of peptides. Essentially identical results were obtained in three other experiments.

Figure 8

Inhibition of DNA Synthesis by pseudosubstrate peptides

Different concentrations of peptides ALR (SEQ.ID NO. 9) and ARR (SEQ.ID No. 1) were microinjected along with a marker antibody into the cytoplasm of serum-starved (24h) Swiss 3T3 fibroblasts. Immediately after microinjection, bromo-deoxyuridine was added diluted 1:1000 in culture medium, and cells were incubated for 20h at 37°. Afterwards, cells were fixed and processed both for immunofluorescence with an anti-marker antibody and for immunochemical analysis with an antibromodeoxyuridine antibody following standard procedures. Results are representative of at least three independent experiments.

Figure 9

Low serum growth of different cell transfectants

Cells were seeded at 4 x 10^4/culture dish (60mm) in DMEM supplemented with 10% FCS; 24 hours later, cells were counted to confirm accurate plating, at

which time medium was removed and cells refed with DMEM containing 0.5% FCS. Cells were then counted every other day. Results are the mean ± SD of three independent experiments with incubations in duplicate.

EXPERIMENTAL PROCEDURES

Oocyte culture

Oocytes were prepared following standard procedures (Garcia de Herreros et al. (1991) loc cit.). Briefly, ovaries from Xenopus laevis frogs (Blades Biologicals, UK) were incubated with 2mg/ml of collagenase (Boehringer Mannheim, Germany) for 45 min in modified Barth Solution (MBS) without Ca^{2+} (110mM NaCl, 2mM KCl, 1mM $MgCl_2$, 1mM $CaCl_2$, 2mM $NaHCO_3$, 10mM HEPES, pH 7.5). After extensively washing, stage VI oocytes were selected and incubated overnight at 20^0C.

cDNA cloning of Xenopus oocyte ζ–PKC

For cDNA cloning of the ζ–PKC homolog in Xenopus oocytes, a probe was generated by amplification of a DNA fragment directly on a lambda-ZAP library from rat brain by the polymerase chain reaction with the following oligonucleotides:

5'-ATGAATTCTGAAGGCGCACTAC-3'(SEQ ID NO. 10) and

5'- ATGAATTCTC GATGACAGGCTTA-3' (SEQ ID NO. 11).

This resulted in a fragment of 656 bp encompassing nucleotides 52 to 708 of rat brain ζ–PKC (Ono et al., Proc. Natl. Acad. Sci. USA 86,3099 (1989)). This fragment was labeled with ^{32}P by random priming (Multiprime DNA labelling System; Amersham Int.) and used to screen an oligo(dT)-primed Xenopus oocyte cDNA library. Hybridizations were carried out in 50% formamide at 42^0C and filters washed at 65^0C with 0.1 x SSC, 0.1% SDS. Positive signals were picked and phages purified. Inserts were subcloned into pBluescript plasmid. Clones were analyzed by DNA-sequencing with fmol™ DNA Sequencing System (Promega, Madison).

In vitro transcription

The bacteriophage T3 or T7 RNA polymerases were used to synthesize sense and antisense RNAs in the presence of the cap analogue GpppG by using 10µg of linearized DNA as template (mCAP™ mRNA capping Kit; Stratagene, CA.

Oligonucleotides

Fifteen-mer oligonucleotides identical or complementary to the beginning (starting at the initiation codon) of the coding region of Xenopus ζ–PKC were synthesized and modified on the backbone to phosphorothioates (Operon Technologies, Alameda, C. As a further control a nonsense oligonucleotide with a random sequence of nucleotides identical to those present in the antisense oligonucleotide was synthesized.

Isolation of PC-PLC from Bacillus cereus

PC-PLC was isolated from cultures of Bacillus cereus SE-1 essentially as described previously (Larrodera et al. (10) loc. cit.); Garcia de Herreros et al (11 loc. cit). Following this protocol the enzyme preparation was purified to complete homogeneity as confirmed by SDS-PAGE followed by silver-staining. The specific activity of the purified enzyme was 1.5 U/µg.

Preparation of ras p21 proteins

Transforming and normal ras p21 proteins were expressed in bacteria as previously described (Dominguez et al., EMBOJ. 10, 3215 (1991)). A final step of purification consisted in a gel filtration chromatography through a 2.5 x 90 cm Sephadex G-100 column; fractions containing the purified protein were pooled and dialyzed extensively against 20mM Tris-HCl pH 7.5 to remove urea and kept at -70^{0}C until utilized.

Immunoblot analyses of different PKC isotypes

Extracts from Xenopus oocytes containing 100µg of total cell protein were resolved in 10% SDA-polyacrylamide gels following denaturation in SDS sample buffer. Afterwards, they were transferred electrophoretically onto polyvinylidine difluoride membrane (Immobilon, Millipore Continental Water Systems, Bedford, MA) and incubated with antibodies specific for PKC isotypes α, ß and γ (A) or ζ (B), either in the absence or in the presence of the corresponding isoenzyme-

specific peptides A or Z (SEQ ID Nos 6 or 5 respectively). PKC isotypes α, ß and γ on the one hand, and ζ, on the other, were visualized with the AuroProbe™ BL plus system (Amersham, Int.) following incubation of blots with the corresponding antibodies. For detection of isotypes α + ß + γ , an anti-peptide antibody generated against the peptide ILKKDVVIQDDDVE (SEQ ID NO. 12) corresponding to aminoacids 381-394 of γ-PKC, was used. For detection of isotype ζ, an anti-peptide antibody generated using the peptide corresponding to aminoacids 577-592 of ζ–PKC (sequence GFEYINPLLLSAEESV, SEQ ID NO. 13) was used. These antibodies were purchased from Givco BRL (Gaithersburg, MD).

Analysis of oocyte maturation

Groups of 20 oocytes were cultured at 20^{0}C in modified Barth solution, and germinal vesicle (nuclear) breakdown (GVBD) was assessed by the appearance of a white spot in the animal pole. In some cases, nuclear breakdown was confirmed by dissection of trichloroacetic acid (10%)-fixed oocytes (Garcia de Herreros et al. (1991) loc. cit.).

Maturation promoting factor histone 1 kinase assay

Twenty oocytes were homogenized in a buffer containing 20mM HEPES (pH 7.0), 10mM ß-glycerophosphate, 5mM EGTA, 5mM $MgCl_2$, 50mM NaF, 2mM dithiothreitol, 100µg of leupeptin/ml, and 100µM phenylmethylsulfonyl fluoride. Following centrifugation at 13000xg for 15min, extracts (1-2mg/assay) were assayed for 10min at 30^{0}C in a final reaction volume of 50µl containing 20mM HEPES (pH 7.0), 5mM ß-mercaptoethanol, 10mM $MgCl_2$, 100µM [γ-^{32}P] (2-5dpm/fmol), 0.2 µg of heat-stable inhibitor of cAMP-dependent protein kinase, and 0.6mg/ml of Sigma type III-S calf thymus histone. Reactions were terminated, spotted onto Whatman p81 phosphocellulose paper, washed and quantitated as described (Garcia de Herreros et al. (1991) loc. cit). In some experiments, extracts were incubated with p13^{suc1} linked to agarose beads, and histone 1 kinase activity was determined in the precipitates followed by separation in SDS-PAGE (Dominguez et al. (1991) loc. cit.).

Immunoprecipitation and autophosphorylation assay

Oocyte extracts were incubated with 10µg of anti-ζ–PKC antibody and immunocomplexes were recovered with protein G-agarose. Immunoprecipitates were assayed for the autophosphorylation kinase activity of ζ–PKC in a mixture of 300µM [γ-^{32}P]ATP with or without 80nM Ca^{2+} either in the absence or in the presence of 50µg/ml phosphatidylserine (PS). Incubations were carried out with different concentrations of peptides A or Z (SEQ ID NOS 6 or 5 respectively). Reactions were stopped by 45min and proteins resolved by SDS-PAGE. No kinase activity was detected when oocyte extracts were incubated with protein G-agarose in the absence of antibody. Essentially identical results were obtained in three independent experiments.

Example 1

Ala-Arg-Arg (Peptide Z4) (SEQ ID NO 1)

Example 2

Ala-Arg-Arg-Trp (Peptide Z3) (SEQ ID NO 2)

Example 3

Ala-Arg-Arg-Trp-Arg (Peptide Z2) (SEQ ID NO 3)

Example 4

Ala-Arg-Arg-Trp-Arg-Lys (Peptide Z1) (SEQ ID NO 4)

Example 5

Arg-Arg-Gly-Ala-Arg-Arg-Trp-Arg-Lys (Peptide Z) (SEQ ID NO 5)

Example 6

N-acetyl-Ala-Arg-Arg (SEQ ID NO. 17)

The peptides of Examples 1 to 6 (SEQ ID NOS 1 to 5 and 17 respectively) were all synthesised using the Merrifield solid support method and were analysed by amino acid analysis and reverse phase HPLC. All peptides had an amino acid analysis as expected according to the structure and were shown to be at least 95% homogeneous (single peak was obtained by reverse phase

HPLC). Peptide concentrations were calculated from the amino acid analysis data.

Example 7

PKC activity in X. laevis oocytes and bovine brain

Experiments with oocytes from Xenopus laevis permit one to investigate the role of different enzymatic activities in mitogenic signals generated in response to the microinjection of different stimuli or inhibitors. In order to assess the functional importance of PKC in the maturation of Xenopus oocytes, we initially determined whether phorbol myristate acetate (PMA), a very well known activator of classical PKC subspecies (Y.Nishizuka, (1988) loc. cit), stimulated maturation promoting H1-kinase activity in oocytes (Garcia de Herreros et al., (1991), loc. cit.). Results from Figure 1 clearly show that addition of PMA up to 200ng/ml produces only a very weak stimulation of H1-kinase activity as compared to that elicited by either microinjection of transforming v-H-ras p21 or microinjection of a permanently activated PC-PLC from B. cereus (Larrodera et al. (1990), loc. cit; Garcia de Herreros et al. (1991), loc. cit.) These results can be interpreted as that either PMA-sensitive PKC activity is not abundant in Xenopus laevis oocytes or, if present, is not directly involved in the activation of H1-kinase.

Accordingly, PKC activity was assayed in extracts from Xenopus oocytes. Results from Table 1 indicate that PKC activity is clearly detectable in oocyte extracts, although its level was significantly lower than in other tissues, like for example bovine brain. (See Table 1, Peptides - None).

TABLE 1

Protein kinase C activity in extracts of Xenopus laevis oocytes and bovine brain.

Stage VI oocytes and bovine brain were homogenized in 20mM Tris-HCl, pH 7.4, 5mM ß-mercaptoethanol, 0.5mM EGTA, 2mM EDTA, 10 µM PMSF, 10µg/ml leupeptin, 1% Triton X-100. After 45 minutes on ice, extracts were centrifuged at 100,000 x g for 30 minutes, after which PKC was purified as described by Nishizuka (1988) loc. cit. Protein kinase C activity was determined under different conditions in 5-10µg of protein extracts using myelin basic protein as phosphate acceptor. Incubations were performed in the presence of Ca^{2+} (100µM) either with or without 100µg/ml of phosphatidylserine (PS) plus PMA (5µg/ml). A mixture of peptides A, D and E corresponding to the PKC isotypes α, ß, γ, (peptide A, SEQ ID NO. 6) δ (peptide D, SEQ ID NO 7) and ε (peptide E, SEQ ID NO 8) was added to some tubes at a final concentration of 5µM. Results are mean ± SD of three independent experiments with incubations in duplicate.

	Xenopus oocytes		Bovine brain	
	dpm/10 min/µg of protein			
Peptides	**None**	**A+D+E**	**None**	**A+D+E**
None	250 ± 15	220 ± 20	650 ± 66	620 ± 50
PS/PMA	720 ± 45	180 ± 16	4300 ± 310	750 ± 40

Example 8

Reconstitution of a PMA-activatable PKC dependent pathway in oocyte maturation

We next attempted to reconstitute a PKC-dependent pathway for the activation of H1-kinase by PMA in the oocyte. Thus, PKC was partially purified from bovine brain following previously standardized protocols and microinjected into Xenopus laevis oocytes. This did not produce any effect on H1-kinase activity (Figure 1); however, incubation of PKC-microinjected oocytes with 100ng/ml of PMA significantly stimulated H1-kinase. Therefore, it is possible to reconstitute a PMA-activatable, PKC-dependent pathway for activation of at least some of the parameters involved in oocyte maturation by microinjecting a purified preparation of PKCs. Accordingly, a PMA-PKC-dependent pathway

does not appear to play a decisive role in oocyte maturation, unless bovine brain PKCs are provided.

Example 9

ζ–PKC involvement in PC-PLC-activated mitogenic pathways

Several kinases have an autoinhibitory region called the pseudosubstrate domain (Soderling, 1990, loc cit). We reason that experiments with oocytes from Xenopus laevis may permit one to investigate the role of different PKC isotypes in mitogenic signalling by microinjection of specific pseudosubstrate peptide inhibitors of the PKC isozymes. Taking into account the PKC isotypes present in oocytes, peptides A, D, E and Z were synthesized. Peptide A (SEQ ID NO. 6) has a sequence conserved in the pseudosubstrate region of PKC isotypes α, ß and γ, and therefore should be a good candidate inhibitor of these isotypes (Osada et al., J. Biol. Chem. 265, 22434 (1990)). Peptides D, E and Z (SEQ ID NOS 7, 8 and 5 respectively) have sequences identical to the PKC pseudosubstrate regions of isoenzyme types δ, ε and ζ respectively, which significantly differ from that of isotypes α, ß or γ (Osada et al. (1990 loc. cit - see legend to Figure 1).

Oocytes were therefore microinjected with a mixture of three peptides corresponding to the conserved pseudosubstrate regions of PKC isotypes α, ß, γ, δ and ε, thought to be good candidate inhibitors of these isotypes, after which they were microinjected with 25μU of B. cereus PC-PLC, or with 20ng of transforming v-H-ras p21, or they were incubated with insulin (1μM) or progesterone (1μM). Control oocytes were microinjected with bovine brain PKC and incubated, afterwards, with 100ng/ml of PMA. Results from Figure 1 clearly demonstrate that the presence of the mixture of pseudosubstrate peptides A, D and E (SEQ ID NOS 6, 7 and 8) did not affect the ability of either progesterone, insulin, transforming v-H-ras p21 or B.cereus PC-PLC to activate H1-kinase although it completely abolished the stimulation of this functional parameter by PMA in PKC-microinjected oocytes. Of note is that the pseudosubstrate mixture completely abolished PKC activity from either bovine brain or Xenopus oocytes, in an in vitro assay system (Table 1). All these data strongly indicate that none of the above mentioned PKC isotypes appear to be implicated in the transmission of the maturation signal by insulin/ras p21/PC-PLC.

The results presented so far are consistent with previous observations demonstrating that down-regulation of PMA-sensitive PKC isotypes by chronic exposure of fibroblasts to phorbol esters did not affect signalling responses to ras p21/PC-PLC (P. Larrodera et al., (1990), loc. cit.; M.T. Diaz-Meco et al J. Biol. Chem. 266, 22597 (1991). One important property of the other PKC isotype present in Xenopus oocytes, namely ζ–PKC, is its lack of sensitivity to PMA (Y.Ono et al., J. Biol. Chem. 263, 6927 (1988) and Y. Ono et al., (1989) loc.cit). Therefore, if a PKC isotype is to play any role in maturation of Xenopus oocytes, the isotype ζ appears to be a good candidate.

In order to test the hypothesis regarding ζ–PKC, we initially cloned the cDNA encoding the Xenopus laevis ζ–PKC homologue. To this aim, we used a cDNA library from Xenopus oocytes and a 656-bp probe generated by polymerase chain reaction from a rat brain cDNA library and the appropriate primers. This probe encompasses the regulatory portion of the enzyme, including the cysteine-rich domain. From the sequence of this clone it is clear that the Xenopus ζ–PKC displays a 92% identity at the aminoacid level to its rat brain homologue. All the important signatures of the enzyme, including the cysteine-rich and ATP-binding domains are perfectly well conserved. Interestingly, the sequence corresponding to the pseudosubstrate region is 100% identical at the aminoacid level to that from its rat brain homologue.

Subsequently, a new PKC isozyme, designated ζnPKC, was isolated from Xenopus oocytes using the same 656-bp rat brain probe. This enzyme is closely related to, but distinct from the Xenopus laevis homologue to rat brain ζ–PKC, and shares great homology with ζ–PKC (73% identity at the amino acid level to its rat brain homologue) and has a similar overall structure. The pseudosubstrate region is perfectly conserved between Xenopus laevis ζ–PKC and ζnPKC and rat brain ζ–PKC.

Results from Figure 1 demonstrate that oocytes microinjected with a synthetic peptide having a sequence identical to the PKC isotype ζ pseudosubstrate region (SEQ ID NO.5) did not respond to the activation with insulin, ras p21, or PC-PLC although they did respond to the addition of progesterone. The ability of PMA to stimulate H1-kinase in PKC-microinjected oocytes was not affected by the microinjection of this peptide. This strongly suggests that ζ–PKC is a specific critical step in mitogenic signal transduction in response to insulin/ras p21/PC-PLC. The fact that the progesterone - activated

maturation program is not affected by this peptide is a good control of the specificity of its effect on the insulin pathway.

Example 10

Identification of PKC isotypes present in extracts of Xenopus laevis oocytes

To determine which specific PKC isotypes were present in stage VI oocytes from Xenopus laevis antibodies specific for each PKC isotype were used in immunoblots of oocyte extracts. Results from Figure 2 (panel A) demonstrate that immunoblotting with an antibody specific for isotypes α, β and γ detected an 80 kDa band in oocyte extracts. Similarly, immunoblotting with an antibody specific for ζ–PKC clearly detected a band with an approximate molecular mass of 65 kDa (Figure 2, panel B); this size is consistent with the sequence data of its cDNA from rat brain (Ono et al., 1989, loc.cit) and from Xenopus oocytes (see Example 9). These bands were specifically eliminated by blockade of the antibody with the corresponding isoenzyme-specific peptides (Figure 2, + peptide). When immunoblotting of oocyte extracts was carried out with antibodies monospecific for isotypes δ and ε, respectively, no bands were observed although these antibodies readily detected the corresponding PKC isotypes in rat brain extracts (not shown).

Example 11

Inhibition of ζ–PKC activity

In order to determine whether peptide Z (SEQ ID NO. 5) actually blocks the enzymatic activity of ζ–PKC, the following experiment was carried out. Oocyte extracts were immunoprecipitated with the specific anti-ζ–PKC antibody described above and autophosphorylation of ζ–PKC was measured in the protein G-agarose recovered immunocomplexes. Results from Figure 3 demonstrate that a dramatic autophosphorylation of ζ–PKC was observed in the immunoprecipitates incubated in the presence of phosphatidylserine. Interestingly, as little as 0.1μM peptide Z (SEQ ID NO. 5) completely abolished ζ–PKC autophosphorylation (Figure 3). A comparable level of inhibition of this activity was detected only with a concentration of peptide A (SEQ ID NO. 6) which was twenty-fold higher than that of peptide Z (SEQ ID NO. 5). These results demonstrate that the inhibition of ζ–PKC by peptide Z (SEQ ID NO. 5) was actually specific.

Example 12

Inhibition of germinal vesicle breakdown (GVBD)

Since activation of H1 kinase is critical for the control of maturation in Xenopus oocytes, conceivably, microinjection of peptide Z (SEQ ID No. 5) will inhibit GVBD in response to the stimulation of the insulin pathway but not to progesterone. Results from Table 2 indicate that this is actually the case.

Thus, microinjection of transforming v-H-ras p21, or B. cereus PC-PLC promote a potent maturation response comparable to that produced by the addition of insulin or progesterone. Addition of PMA does not induce GVBD unless oocytes were previously microinjected with purified bovine brain PKC, consistent with the data on H1 kinase. Of note is that microinjection of peptide A (SEQ ID NO 6) inhibited PMA-induced maturation in PKC-microinjected oocytes but produced little or no effect on the induction of GVBD in response to insulin/ras p21/PC-PLC or progesterone. Interestingly, microinjection of peptide Z (SEQ ID NO 5) completely abolished GVBD in response to microinjection of transforming v-H-ras p21, B.cereus PC-PLC or to the addition of insulin. It is noteworthy that GVBD induction in response to progesterone or PMA, in PKC-microinjected oocytes, were not affected by microinjection of peptide Z (SEQ ID NO 5).

TABLE 2

Effect of pseudosubstrate peptides A and Z (SEQ ID NOS 6 and 5 respectively) on maturation of Xenopus laevis oocytes.

	Control	Peptide A	Peptide Z
		GVBD (%)	
None	0	0	0
Insulin	65 ± 8	68 ± 6	20 ± 2
Progesterone	89 ± 6	90 ± 8	88 ± 9
PKC (brain)	8 ± 2	2 ± 1	9 ± 2
PKC + PMA	45 ± 8	4 ± 2	40 ± 4
ras p21	80 ± 7	78 ± 6	12 ± 5
PC-PLC	74 ± 9	70 ± 8	14 ± 9

Groups of 20 oocytes were cultured at 20^{0}C in modifed Barth solution following different treatments and germinal vesicle (nuclear) breakdown (GVBD) was assessed by the appearance of a white spot in the animal pole. In some cases, nuclear breakdown was confirmed by dissection of trichloroacetic acid (10%)-fixed oocytes. Oocytes were either microinjected with buffer control or with 1 ng of purified bovine PKC. Afterwards, corresponding oocytes were microinjected with either water, or peptides A or Z (5 µM final concentration into the oocyte), respectively. Subsequently, they were either incubated in the presence of PMA (100 ng/ml), insulin (1 µM) or progesterone (1µM), or microinjected with transforming v-H-ras p21 (20 ng) or with B.cereus PC-PLC (25µU) and GVBD determined 6h thereafter. Results are mean ± SD of three independent experiments with incubations in duplicate.

Example 13

Effect of antisense RNA from ζ-PKC on the activation of maturation promoting factor H1-kinase in Xenopus oocytes

In order to further demonstrate the importance of ζ-PKC in oocyte maturation, a fragment of the regulatory domain of this PKC isotype, encompassing bases -10 to +600, was subcloned into pBluescript. Sense and antisense RNAs were synthesized in vitro from that plasmid and both RNAs

were microinjected into stage VI oocytes. At different times following microinjection, they were extracted and the levels of different PKC isotypes were determined by immunoblotting. Results from Figure 4 demonstrate that 48h after microinjection of 25 ng of antisense ζ–PKC RNA, a significant reduction in the level of this protein is observed, with no effect on the immunoreactive band detected with the antibody specific for isotypes α, ß and γ. Therefore, by using this strategy we have been able to specifically deplete ζ–PKC levels in oocytes. Microinjection of water or control sense RNA did not produce any effect on either PKC isotypes (Figure 4).

Accordingly, oocytes incubated for 48h with either sense or antisense ζ–PKC RNAs were microinjected with 25µU of B.cereus PC- PLC, or with 20ng of transforming v-H-ras p21, or they were incubated with insulin (1µM) or progesterone (1µM). Results from Figure 5A clearly demonstrate that ζ–PKC-depleted oocytes responded less efficiently to the activation with insulin, ras p21, or PC-PLC although they gave a full response to the addition of progesterone. This strongly suggests that ζ–PKC is a specific critical step in maturation signal transduction in response to insulin/ras p21/PC- PLC. The fact that the progesterone-activated maturation program is not affected by depletion of ζ–PKC is a good control of the specificity of this effect on the insulin pathway. Control oocytes microinjected with either water or sense RNA, were microinjected in parallel with transforming v-H-ras or PC-PLC or incubated with insulin or progesterone (Figure 5A). Microinjection of water or ζ–PKC sense RNA did not affect the ability of these molecules to activate H1-kinase.

Example 14

Effect of small antisense oligonucleotides on the activation of maturation promoting factor H1 kinase in Xenopus oocytes

The use of small antisense oligonucleotides is a potential method utilized to inhibit the expression of proteins in oocytes (Sumikawa and Miledi, Proc. Natl. Acad. Sci. USA 85,1302 (1988)). Therefore, as an independent strategy to deplete ζ–PKC levels in oocytes we synthesized a 15-mer antisense oligonucleotide starting at the initiation codon of ζ–PKC as well as the corresponding sense and nonsense controls. To overcome degradation by nucleases, the oligonucleotides were modified on the backbone to

phosphorothioates (Matsukura et al., Proc. Natl. Acad, Sci. USA (1987), 84(21), 7706; Agrawal et al., Proc. Natl. Acad. Sci. USA (1988), 85(19), 7079). Oocytes were microinjected with 150ng of either sense (SEQ ID NO 15), antisense (SEQ ID NO 14) or nonsense oligonucleotides. A significant depletion of ζ–PKC levels was detected in western blots of extracts from antisense oligonucleotide-microinjected oocytes with no effect on the other PKC isotypes (not shown). Microinjection of sense or nonsense oligonucleotides did not affect the levels of any PKC isotype present in oocytes. Therefore, oocytes were microinjected with 25μU of B.cereus PC-PLC, or with 20ng of transforming v-H-ras p21, or they were incubated with insulin (1 μM) or progesterone (1μM). Results from Figure 5B clearly demonstrate that the ability of ζ–PKC-depleted oocytes to respond to insulin, ras p21, or PC-PLC was significantly impaired although they did respond normally to the addition of progesterone. Microinjection of sense or nonsense oligonucleotides did not affect the activation of H1 kinase by any of the stimuli tested in this study.

Example 15

Effect of ζ–PKC depletion on GVBD in Xenopus oocytes

Depletion of ζ–PKC levels in Xenopus oocytes will conceivably lead to the inhibition of GVBD induction in response to insulin/ras p21/PC-PLC but not to progesterone. Results from Table 3 indicate that this is actually the case.

Thus, microinjection of transforming v-H-ras p21, or B.cereus PC-PLC promote a potent maturation response comparable to that produced by the addition of insulin or progesterone. Interestingly, depletion of ζ–PKC levels by microinjection of its antisense RNA, dramatically inhibited GVBD induction in response to microinjection of transforming v-H-ras p21, B.cereus PC-PLC or to the addition of insulin. A good control of the specificity of this effect is that GVBD induction in response to progesterone was not affected by ζ–PKC depletion.

TABLE 3.

Maturation of Xenopus laevis oocytes

	Control	ζ–PKC-depleted
	GVBD (%)	
None	0	0
Insulin	65 ± 8	35 ± 2
Progesterone	89 ± 6	88 ± 9
ras p21	80 ± 7	35 ± 5
PC-PLC	74 ± 9	38 ± 9

Groups of 20 oocytes either control or with ζ–PKC levels depleted by microinjection of antisense RNA were cultured at 20^0C in modified Barth solution following different treatments, and germinal vesicle (nuclear) breakdown (GVBD) was assessed by the appearance of a white spot in the animal pole. In some cases, nuclear breakdown was confirmed by dissection of trichloroacetic acid (10%)-fixed oocytes. They were incubated in the presence of PMA(100 ng/ml), insulin (1 μM) or progesterone (1μM), or microinjected with transforming v-H-ras p21 (20ng) or with B.cereus PC-PLC (25 μU) and induction of GVBD determined 6h thereafter. Results are mean ± SD of three independent experiments with incubations in duplicate. ND, not determined.

Example 16

Other pseudosubstrate inhibitors of ζ–PKC

In order to be able to initiate a process that eventually could lead us to a rational design of potential inhibitors of this kinase, a comparison of the aminoacid sequences of the different PKC pseudosubstrate regions was carried out. From Figure 6 it is clear that the three first aminoacids in these sequences are the most conserved. Thus, it can be assumed that this part of the molecule is unlikely to confer specificity to potential peptide inhibitors. Therefore, we synthesized peptide Z1 (SEQ ID NO.4) which lacks the three first aminoacids, and its ability to inhibit H1-kinase stimulation was investigated. Results shown in Figure 7 demonstrate that microinjection of Z1 (SEQ ID NO. 4)into Xenopus

oocytes blocks the activation of H1-kinase to an extent comparable to that produced by the full-length Z pseudosubstrate (SEQ ID NO.5).

Peptides were then synthesized with deletions in the C-terminus of Z1 (SEQ ID NO 4) to give pseudosubstrates Z2, Z3 and Z4 (Figure 6) (SEQ ID NOS. 3, 2 and 1 respectively). Interestingly, even a tripeptide with the sequence ARR (Z4, SEQ ID NO.1) was capable of inhibiting the activation of H1-kinase (Figure 7). As a control of specificity, the tripeptide ALR (A4, Figure 6) (SEQ ID NO.9) was synthesized. This peptide has the sequence corresponding to deletions in the pseudosubstrate A which is specific for PKC isotypes α, ß and γ (see Figure 6). Results from Figure 7 indicate that microinjection of peptide A4 (SEQ ID NO. 9) does not affect activation of H1-kinase.

In order to address the ability of peptides with sequences included in the pseudosubstrate region of ζ–PKC to inhibit growth of mammalian somatic cells, the following experiments were carried out.

Example 17

ζ–PKC pseudosubstrates inhibit growth of mouse NIH-3T3 fibroblasts

Peptide Z1 (SEQ ID NO. 5) was microinjected into mouse NIH-3T3 fibroblasts growing in the presence of 10% fetal calf serum, and DNA synthesis was determined as described in the legend to Table 4. As a control a nine-aminoacid peptide A (SEQ ID No. 6) with a sequence corresponding to the pseudosubstrate region of PKC isotypes α, ß and γ (see Figure 1) was also microinjected in parallel cultures. Results from Table 4 demonstrate that microinjection of peptide Z1 (SEQ ID NO 5) dramatically inhibits DNA synthesis in proliferating NIH-3T3 fibroblasts, while peptide A (SEQ ID NO. 6) produced little or no effect.

TABLE 4

Inhibition of entry into DNA synthesis by a pseudosubstrate peptide inhibitor specific for ζ–PKC

	Incorporation of bromo-deoxyuridine
Cells injected with :	Number of cells synthesizing DNA/microinjected cells
Control buffer	22/24 (92%)
Peptide A	20/24 (83%)
Peptide Z	11/33 (33%)

Peptides A or Z (SEQ ID NOs 6 or 5 respectively) were microinjected along with a marker antibody into the cytoplasm of growing NIH-3T3 fibroblasts about 8-10h following serum refeeding (beginning of S phase). Immediately after microinjection, bromo-deoxyuridine (Amersham International), an analogue of thymidine, was diluted 1:1000 in culture medium, and cells were grown at 37^0 for 24hr. Cells were fixed and either immunofluorescence processing with anti-marker antibody or immunochemical detection of bromo-deoxyuridine incorporation was done following standard procedures. Results are representative of another two with identical or similar results.

Example 18

ζ–PKC pseudosubstrates inhibit growth of Swiss - 3T3 fibroblasts

Different peptides were microinjected into Swiss-3T3 fibroblasts growing in the presence of 10% fetal calf serum. Cells were synchronized by serum starvation 24h before microinjection, and DNA synthesis was determined as described in the legend to Table 5.

Results from Table 5 demonstrate that microinjection of peptides based on the sequence of the pseudosubstrate region of ζ–PKC dramatically inhibited DNA synthesis in serum-stimulated Swiss 3T3 fibroblasts whereas peptides corresponding to the pseudosubstrate region of PKC isotypes α, b and γ inhibited DNA synthesis much less potently.

A dose-response curve of tripeptides ALR (SEQ.ID.NO.9) and ARR (SEQ.ID.NO.1) is shown in Figure 8. These results demonstrate that DNA

synthesis in at least 80% of Swiss 3T3 fibroblasts is inhibited by as little as 1-2μM inhibitory peptide ARR (SEQ. ID. NO. 1) under the conditions utilised. In contrast peptide ALR (SEQ. ID. NO. 9) inhibits DNA synthesis in not more than 50% of such cells at concentrations ten-fold higher (16μM). This demonstrates the potent and selective inhibitory properties of the peptide according to the invention.

TABLE 5

Inhibition of DNA synthesis by pseudosubstrate peptide inhibitors specific for ζ-PKC

	Incorporation of bromo-deoxyuridine
Cells injected with peptides[X]	number of cells synthesizing DNA/microinjected cells
None (control buffer)	129/150 (86%)
RKGALRQKN (SEQ ID NO.6)	77/150 (51%)
RRGARRWRK (SEQ ID NO.5)	6/150 (4%)
ALR (SEQ ID NO. 9)	75/150 (50%)
ARR (SEQ ID NO. 1)	21/150 (14%)
AcALR (SEQ ID NO. 16)	60/150 (40%)
AcARR (SEQ ID NO. 17)	12/150 (8%)

Different peptides were microinjected along with a marker antibody into the cytoplasm of serum-starved (24h) Swiss 3T3 fibroblasts (beginning of G1 phase). Immediately after micoinjection, bromo-deoxyuridine was added diluted 1:1000 in culture medium, and cells were incubated for 20h at 38°. Afterwards, cells were fixed and processed both for immunofluorescence with an anti-marker antibody and for immunochemical analysis with an anti-bromodeoxyuridine antibody following standard procedures. Results are representative of at least three independent experiments.

[X] The concentration of peptides into the microinjection pipette was 8μM. A 20 to 50-fold dilution occurs once the molecules enter the cell.

Example 19

ζ-PKC pseudosubstrates inhibit growth of human umbilical endothelial cells

Different peptides were microinjected into human umbilical endothelial cells (HUVECs) growing in the presence of 10% fetal calf serum. Cells were synchronized by serum starvation 24h before microinjection, and DNA synthesis was determined as described in the legend to Table 6.

The results from Table 6 confirm the results of Example 18.

TABLE 6

Inhibition of DNA synthesis by pseudosubstrate peptide inhibitors specific for ζ-PKC

	Incorporation of bromo-deoxyuridine
Cells injected with peptides[Y]	number of cells synthesizing DNA/microinjected cells
None (control buffer)	90/100 (90%)
RKGALRQKN (SEQ ID NO.6)	51/100 (51%)
RRGARRWRK (SEQ ID NO.5)	16/100 (16%)
ALR (SEQ ID NO. 9)	56/100 (56%)
ARR (SEQ ID NO. 1)	25/100 (25%)

[Y] The concentration of peptides into the microinjection pipette was 8μM. A 20 to 50-fold dilution occurs once the molecules enter the cell.

Different peptides were microinjected along with a marker antibody into the cytoplasm of serum-starved (24h) HUVECs (beginning of G1 phase). Immediately after microinjection; bromo-deoxyuridine was added diluted 1:1000 in culture medium, and cells were incubated for 20h at 37^0. Afterwards, cells were fixed and processed both for immunofluorescence with an anti-marker antibody and for immunochemical analysis with an anti bromo-deoxyuridine antibody following standard procedures. Results are representative of at least three independent experiments.

Example 20

Alterations in growth properties by overexpression of ζ–PKC

The growth properties of NIH-3T3 fibroblast cell lines overexpressing ζ–PKC were determined. The cDNA of ζ–PKC was subcloned into a mammalian expression vector under the control of a potent transcriptional promoter (pRcCMV-Invitrogen, USA) and a comparison of the growth rate of ζ–PKC-overexpressing clones (pRcCMVζ4 and pRcCMVζ6) was carried out under low-serum conditions (0.5% FCS), using a ras-transformed cell line as a positive control and the corresponding negative controls. Results from Fig.9 show that clones pRcCMVζ4 and pRcCMVζ6 displayed a dramatically increased degree of growth at 0.5% serum which is similar to that of the ras transformed cell line. This indicates that overexpression of ζ–PKC decreases the requirement of mitogens for cell proliferation. Furthermore, according to the data from TABLE 7 both transfectants displayed lower doubling times and higher saturation densities as compared to controls.

Table 7

Growth properties of cells overexpressing ζ–PKC

	Growth in monolayers	
Cell line	Doubling time (hours)	Saturation density (10^6 cells)
NIH-3T3	26.4 ± 1.5	3.1 ± 0.3
pRcCMV	25.8 ± 1.2	3.4 ± 0.4
pRcCMVζ4	23.5 ± 0.9	5.9 ± 0.4
pRcCMVζ6	21.4 ± 1.4	7.2 ± 0.5

The growth in monolayers was measured by plating 4 x 10^4 cells/culture dish (35mm) in DMEM supplemented with 10% FCS (fetal calf serum). Medium was changed every other day. Doubling time was determined by counting cells every 2 days. Saturation density was the number of cells in culture 7 days after reaching confluence. Results are mean ± SD of three independent experiments with incubations in triplicate.

These results suggest that the simple overexpression of ζ–PKC induces some of the characteristics of the transformed phenotype, which is consistent with the notion that this enzyme is a critical event in mitogenic signalling.

Example 21

Sensitive autophosphorylation assay

A sensitive autophosphorylation assay to determine ζ-PKC activity was developed by deletion of the regulatory domain of cloned rat brain ζ-PKC using conventional recombinant DNA techniques. The cDNA of the permanently active ζ-PKC mutant thus produced was subcloned into the PMALc2 plasmid to obtain a recombinant fusion protein (MBP-ζPKCdel). Bacterial cultures containing this plasmid were induced and the protein purified by affinity chromatography on an amylose-sepharose column. ζ-Protein kinase C activity was determined as described hereinbefore using myelin basic protein as phosphate acceptor. Typical assay conditions were final reaction volume 40µl, enzyme 2µl, buffer (HEPES 50mM, $MgCl_2$ 10mM, $CaCl_2$ 1mM, EGTA 1mM, ATP 10µM final concentrations), ATP-α-^{32}P 1µCi, myelin basic protein 2µg, potential inhibitor e.g. peptide 1µM. Incubations at 30°C may be stopped after e.g. 10 mins by boiling in SDS sample buffer and the sample fractionated e.g. in SDS-PAGE. Gels were dried and exposed for two days.

Spots corresponding to the phosphorylated protein were quantitated by laser scanning densitometry.

Permanently activated ζ-PKC assays are at least ten times more sensitive than autophosphorylation assays described herein utilising native ζ-PKC.

Typical results representative of three independent experiments are shown in Table 8.

Table 8

Inhibition of in vitro ζ-PKC activity by pseudosubstrate inhibitors.

Additions	Relative ζ-PKC activity
None	++++
Peptide Z (SEQ.ID.NO.5)1μM	+
Peptide A (SEQ.ID.NO.6)1μM	+++

Discussion and conclusions

From all the results shown here it appears that PKC isoenzymes α, β and γ are not involved in the maturation pathway activated by insulin/ras p21/PC-PLC. Neither δ nor ε PKC isotypes appear to be involved, since no detectable amounts of these subspecies were found in stage VI oocytes. Consistent with these data is the fact that screening the oocyte cDNA library utilized to clone the Xenopus homologue of ζ–PKC with probes specific for isotypes δ or ε, reveal the complete absence of these PKC isotypes in Xenopus oocytes. Furthermore, microinjection of peptide pseudosubstrate inhibitors corresponding to PKC subspecies δ and ε, produced little or no effect on maturation or H1 kinase induction in response to any of the stimuli described in this study. Interestingly, all these isotypes are activated by PMA and are also down-regulatable by chronic treatment with phorbol esters (Nishizuka, 1988; Ono et al., 1989 loc. cit). The data presented here is consistent with our recent evidence that depletion of PKC levels by long-term incubation of fibroblasts with PMA does not affect the ability of PC-PLC to induce DNA synthesis (Larrodera et al. (1990) loc cit.) or the capability of ras p21/PC-PLC to activate stromelysin gene expression (Diaz–Meco et al. (1991) loc cit).

Thus, it is shown here that PMA is unable to promote maturation in Xenopus oocytes unless a partially purified fraction of PKC from rat brain has previously been microinjected. Immunoblot analysis of this PKC preparation reveals a band recognised by an antibody specific for isotypes α, β and γ, as well as bands detected with antibodies specific for isotypes δ and ε. Therefore, the ability of

PMA to induce maturation under these conditions may be due to an overload of PKC isotypes a+β+γ, or to the presence of isotypes δ and ε which are ordinarily absent in Xenopus oocytes. A new distantly related member of the PKC family of isoenzymes, ζ–PKC, displays a number of interesting characteristics and a relatively detailed analysis of the biochemical properties of this PKC isotype shows that it does not bind PMA (Ono et al. (1989) loc cit), is not stimulated by this pharmacological agent, and is resistant to down-regulation by phorbol esters. In this regard, it is noteworthy that recent studies are beginning to characterize a PKC from yeast which, like ζ–PKC, is insensitive to PMA, and genetic studies strongly suggest that it may play a critical role in cell cycle control (Levin et al., Cell 62, 213 (1990); Ogita et al., Proc. Natl. Acad. Sci. USA 87, 5011 (1990); and Osada et al,(1990) loc cit). Remarkably, we show here that depletion of ζ–PKC subspecies or microinjection of a peptide inhibitor with a sequence specific for the pseudosubstrate region of that PKC isotype inhibited the ability of insulin/ras p21/PC-PLC to activate oocyte maturation with no effect on the pathway activated by progesterone. Therefore, this enzyme seems to play a critical role in the control of proliferative cascades.

Our results strongly suggest that :

1) ζ–PKC is a critical step in the transduction of mitogenic signals funnelled by ras p21/PC-PLC specific pathway;

2) peptides of formula (I) corresponding to the pseudosubstrate region of ζ–PKC, in particular the tripeptide with the sequence ARR, (SEQ ID NO. 1) specifically block mitogenic activation in response to that pathway; and

3) the inhibitory effects of ζ–PKC inhibitory peptides following microinjection into mammalian cells, in particular human cells (e.g. DNA synthesis is severely impaired), provides strong evidence for an anti-proliferative effect in vivo.

Sequence Listing : SEQ ID NOS 1-17

SEQ ID NO: 1
SEQUENCE LENGTH: 3 Amino acids
SEQUENCE TYPE: Amino acid
TOPOLOGY: Linear
MOLECULE TYPE: Peptide
SEQUENCE DESCRIPTION: Ala Arg Arg
1

SEQ ID NO: 2
SEQUENCE LENGTH: 4 Amino acids
SEQUENCE TYPE: Amino acid
TOPOLOGY: Linear
MOLECULE TYPE: Peptide
SEQUENCE DESCRIPTION: Ala Arg Arg Trp
1

SEQ ID NO: 3
SEQUENCE LENGTH: 5 Amino acids
SEQUENCE TYPE: Amino acid
TOPOLOGY: Linear
MOLECULE TYPE: Peptide
SEQUENCE DESCRIPTION: Ala Arg Arg Trp Arg
1 5

SEQ ID NO: 4
SEQUENCE LENGTH: 6 Amino acids
SEQUENCE TYPE: Amino acid
TOPOLOGY: Linear
MOLECULE TYPE: Peptide
SEQUENCE DESCRIPTION: Ala Arg Arg Trp Arg Lys
1 5

SEQ ID NO: 5
SEQUENCE LENGTH: 9 Amino acids
SEQUENCE TYPE: Amino acid
TOPOLOGY: Linear
MOLECULE TYPE: Peptide
SEQUENCE DESCRIPTION: Arg Arg Gly Ala Arg Arg Trp Arg Lys
1 5

SEQ ID NO: 6
SEQUENCE LENGTH: 9 Amino acids
SEQUENCE TYPE: Amino acid
TOPOLOGY: Linear
MOLECULE TYPE: Peptide
SEQUENCE DESCRIPTION: Arg Lys Gly Ala Leu Arg Gln Lys Asn
1 5

SEQ ID NO: 7
SEQUENCE LENGTH: 9 Amino acids
SEQUENCE TYPE: Amino acid
TOPOLOGY: Linear
MOLECULE TYPE: Peptide
SEQUENCE DESCRIPTION: Arg Arg Gly Ala Ile Lys Gln Ala Lys
1 5

SEQ ID NO: 8
SEQUENCE LENGTH: 9 Amino acids
SEQUENCE TYPE: Amino acid
TOPOLOGY: Linear
MOLECULE TYPE: Peptide
SEQUENCE DESCRIPTION: Arg Gln Gly Ala Val Arg Arg Arg Val
1 5

SEQ ID NO: 9
SEQUENCE LENGTH: 3 Amino acids
SEQUENCE TYPE: Amino acid
TOPOLOGY: Linear
MOLECULE TYPE: Peptide
SEQUENCE DESCRIPTION: Ala Leu Arg
1

SEQ ID NO: 10
SEQUENCE LENGTH: 22 nucleotides
SEQUENCE TYPE: Nucleotide
STRANDEDNESS: Single
TOPOLOGY: Linear
MOLECULE TYPE: Other nucleic acid (synthetic)
ANTI-SENSE: No
SEQUENCE DESCRIPTION: ATGAATTCTG AAGGCGCACT AC

SEQ ID NO: 11
SEQUENCE LENGTH: 23 nucleotides
SEQUENCE TYPE: Nucleotide
STRANDEDNESS: Single
TOPOLOGY: Linear
MOLECULE TYPE: Other nucleic acid (synthetic)
ANTI-SENSE: No
SEQUENCE DESCRIPTION: ATGAATTCTC GATGACAGGC TTA

SEQ ID NO: 12
SEQUENCE LENGTH: 14 amino acids
SEQUENCE TYPE: Amino acid
TOPOLOGY: Linear
MOLECULE TYPE: Peptide
SEQUENCE DESCRIPTION: Ile Leu Lys Lys Asp Val Val Ile Gln Asp Asp
1 5 10
Asp Val Glu

SEQ ID NO: 13
SEQUENCE LENGTH: 16 amino acids
SEQUENCE TYPE: Amino acid
TOPOLOGY: Linear
MOLECULE TYPE: Peptide
SEQUENCE DESCRIPTION: Gly Phe Glu Tyr Ile Asn Pro Leu Leu Leu Ser Ala Glu Glu Ser Val
1 5 10 15

SEQ ID NO: 14
SEQUENCE LENGTH: 15 nucleotides
SEQUENCE TYPE: Nucleotide
STRANDEDNESS: Single
TOPOLOGY: Linear
MOLECULE TYPE: Other nucleic acid (synthetic)
ANTI-SENSE: Yes
SEQUENCE DESCRIPTION: GGTCCTGCTG GGCAT

SEQ ID NO: 15
SEQUENCE LENGTH: 15 nucleotides
SEQUENCE TYPE: Nucleotide
STRANDEDNESS: Single
TOPOLOGY: Linear
MOLECULE TYPE: Other nucleic acid (synthetic)
ANTI-SENSE: No
SEQUENCE DESCRIPTION: ATGCCCAGCA GGACC

SEQ ID NO:	16
SEQUENCE LENGTH:	3 amino acids
SEQUENCE TYPE:	Amino acid
TOPOLOGY:	Linear
MOLECULE TYPE:	Peptide
SEQUENCE DESCRIPTION:	Xaa Leu Arg 1
FEATURE:	
NAME/KEY:	Misc.feature
LOCATION:	1
OTHER INFORMATION:	Xaa is N-acetylalanine

SEQ ID NO:	17
SEQUENCE LENGTH:	3 amino acids
SEQUENCE TYPE:	Amino acid
TOPOLOGY:	Linear
MOLECULE TYPE:	Peptide
SEQUENCE DESCRIPTION:	Xaa Arg Arg 1
FEATURE:	
NAME/KEY:	Misc.feature
LOCATION:	1
OTHER INFORMATION:	Xaa is N-acetylalanine

Claims

1. Peptides of general formula (I)

X-Ala-Arg-Arg-J (I)

wherein X is H or one or more amino acids and J is OH or one or more amino acids, and pharmaceutically acceptable derivatives thereof, which peptides contain 3 to 15 amino acid residues.

2. Peptides as claimed in Claim 1 which contain 3 to 9 amino acid residues.

3. Peptides as claimed in Claim 1 or 2 in which X is H, acetyl, Gly, Arg-Gly or Arg-Arg-Gly and J is OH, Trp, Trp Arg or Trp Arg Lys.

4. Peptides as claimed in any one of Claims 1 to 3 which have the formula:
Ala-Arg-Arg (SEQ ID NO. 1);
Ala-Arg-Arg-Trp (SEQ ID NO. 2);
Ala-Arg-Arg-Trp-Arg (SEQ ID NO. 3);
Ala-Arg-Arg-Trp-Arg-Lys (SEQ ID NO. 4);
Arg-Arg-Gly-Ala-Arg-Arg-Trp-Arg-Lys (SEQ ID NO. 5);
and pharmaceutically acceptable derivatives thereof.

5. N-acetyl-Ala-Arg-Arg (SEQ. ID NO. 17) and pharmaceutically acceptable derivatives thereof.

6. Peptides as claimed in any one of claims 1 to 5 in substantially pure form.

7. A method for inhibiting cellular ζ–PKC activity which comprises contacting cells capable of responding to ζ–PKC activation with an effective amount of an inhibitor of ζ–PKC.

8. A method for inhibiting cellular ζ–PKC activity which comprises contacting cells capable of responding to ζ–PKC activation with an effective amount of a peptide as claimed in any one of claims 1 to 6.

9. A screening method for the selection of agents suitable for the prevention or treatment of pathological conditions mediated by ζ–PKC activity which comprises

a) incubating a sample comprising the agent to be tested with an assay system capable of indicating inhibition and/or activation of ζ–PKC activity;

b) determining whether and, if desired, the extent to which ζ–PKC activity is altered by said agent; and

c) selecting agents determined to be potent and selective ζ–PKC inhibitors.

10. A screening method as claimed in claim 9 wherein the agents selected are suitable for the treatment of tumours, hyperproliferative disorders and viral infections.

11. Peptides as claimed in any one of Claims 1 to 6 or physiologically acceptable derivatives thereof for use as active therapeutic agents.

12. Peptides as claimed in any one of Claims 1 to 6 or physiologically acceptable derivatives thereof for use in the treatment of conditions whose underlying aetiology is associated with ζ–PKC activity.

13. Peptides as claimed in any one of Claims 1 to 6 or physiologically acceptable derivatives thereof for use in the treatment of tumours, hyperproliferative disorders or viral infection.

14. Pharmaceutical compositions comprising a peptide as claimed in any one of Claims 1 to 6 or a physiologically acceptable derivative thereof together with one or more physiologically acceptable carriers.

15. Antisense oligonucleotides and physiologically acceptable derivatives thereof corresponding to the DNA coding for ζ–PKC, preferably the regulatory domain of ζ–PKC DNA, or a degenerate equivalent thereof.

16. The oligonucleotide having the sequence GGTCCTGCTGGGCAT (SEQ ID NO 14) or a physiologically acceptable derivative or a degenerate equivalent thereof.

17. Oligonucleotide derivatives as claimed in claim 15 or claim 16 or a degenerate equivalent thereof wherein the backbone is phosphorothioate modified.

18. Pharmaceutical compositions comprising an antisense oligonucleotide as claimed in any one of claims 15 to 17 or a physiologically acceptable derivative or degenerate equivalent thereof together with one or more physiologically acceptable carriers.

19. A process for preparing a peptide as claimed in any one of claims 1 to 6 which comprises

(a) binding the desired protected carboxy-terminal amino acid to a suitable solid support;

(b) reacting other protected amino acids with the support-bound carboxy-terminal amino acid in the desired sequence; and

(c) removing the protecting groups and releasing the peptides so-obtained from the solid support.

20. A method for inhibiting the activity of ζ–PKC in a mammal comprising administration of an effective amount of a peptide as claimed in any one of claims 1 to 6 or a physiologically acceptable derivative thereof.

21. A method for inhibiting the activity of ζ–PKC in a mammal comprising administration of an effective amount of an oligonucleotide as claimed in any one of claims 15 to 17.

22. A method of treating a patient susceptible to or suffering from tumour, hyperproliferative disorder or viral infection which comprises administering to the patient an effective amount of a ζ–PKC inhibitor.

23. A method as claimed in claim 22 wherein said ζ–PKC inhibitor is a peptide as claimed in any one of claims 1 to 6.

24. A method as claimed in claim 22 wherein said ζ–PKC inhibitor is an oligonucleotide as claimed in any one of claims 15 to 17.

FIG. 1

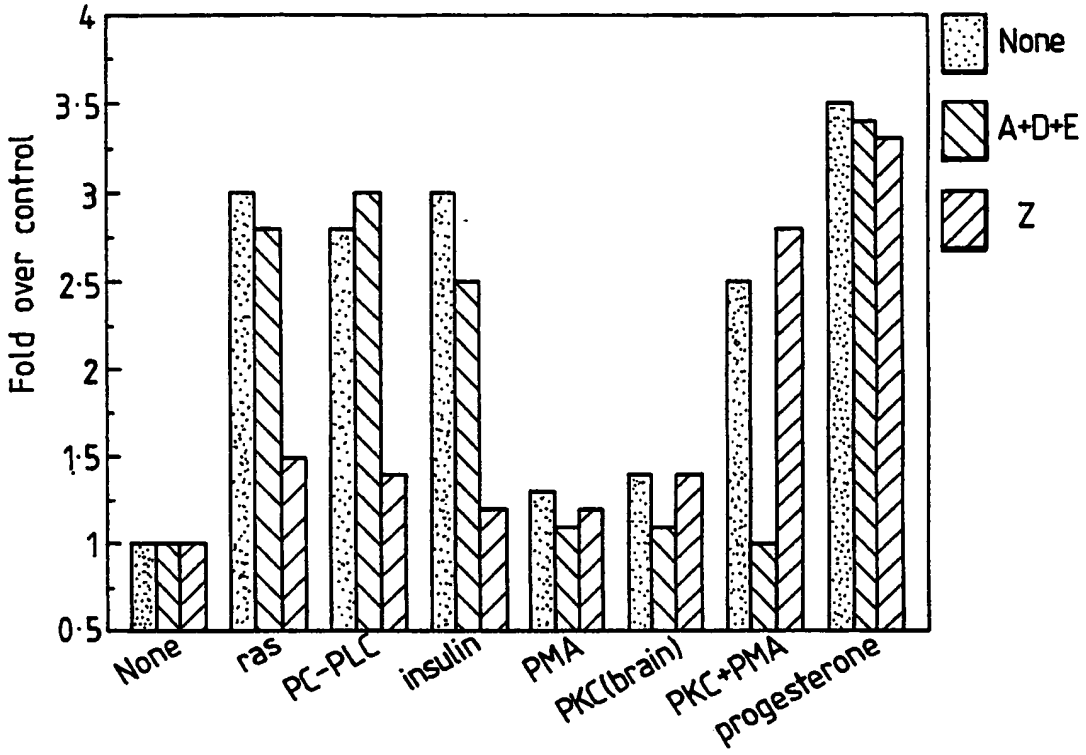

Fold over control
4
3·5
3
2·5
2
1·5
1
0·5
None
ras
PC-PLC
insulin
PMA
PKC(brain)
PKC+PMA
progesterone
None
A+D+E
Z

FIG. 7

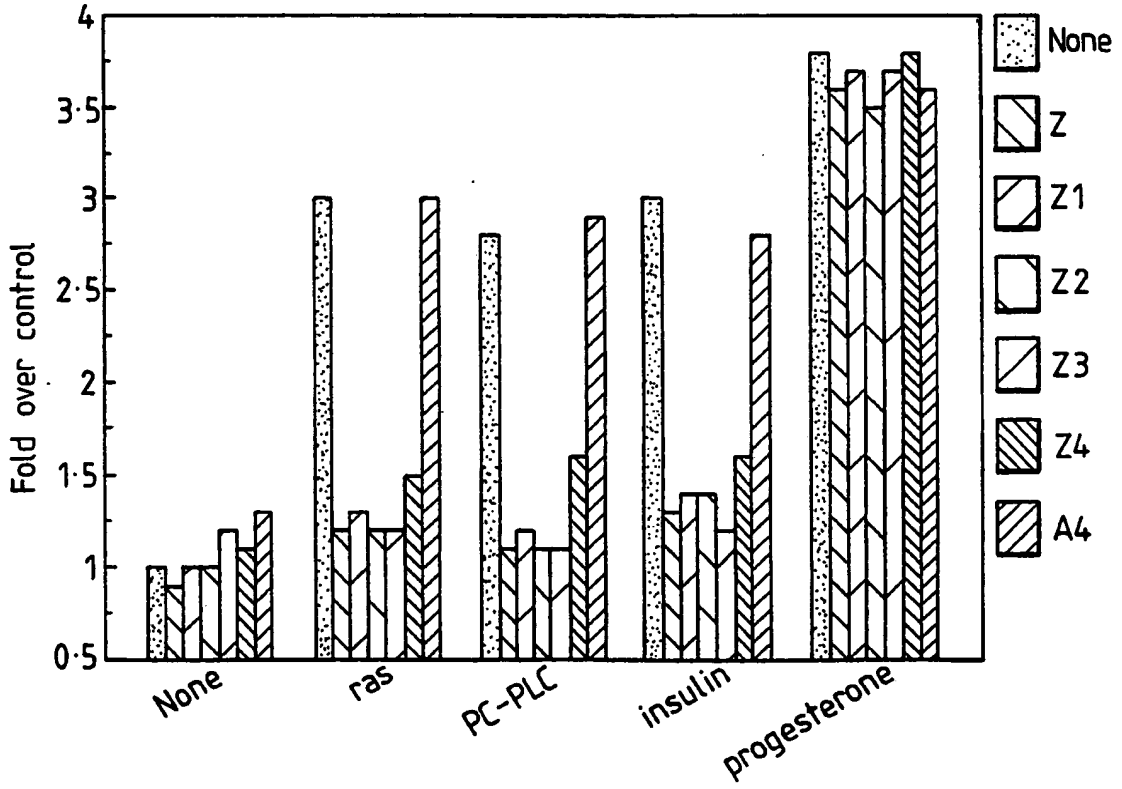

Fold over control
4
3·5
3
2·5
2
1·5
1
0·5
None
ras
PC-PLC
insulin
progesterone
None
Z
Z1
Z2
Z3
Z4
A4

FIG.2

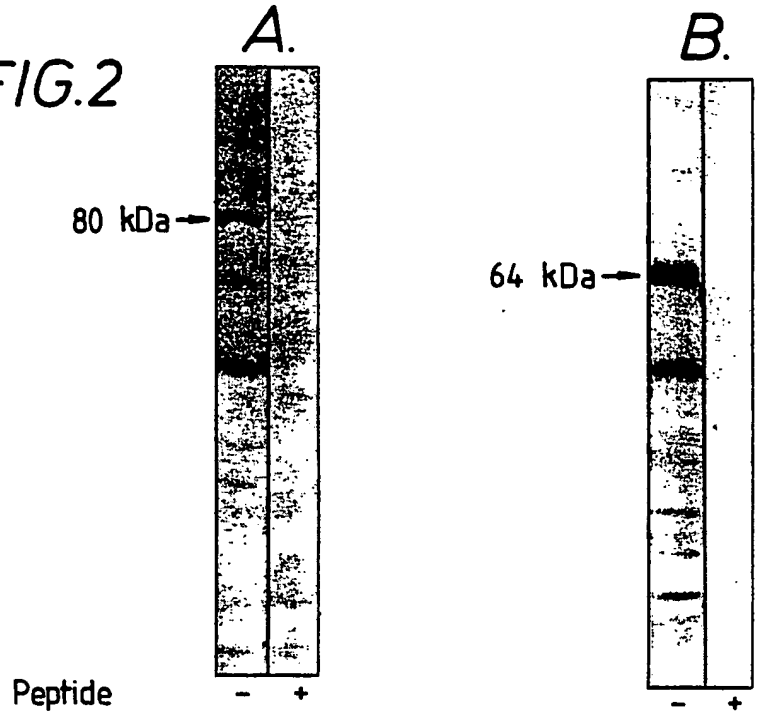


FIG. 3

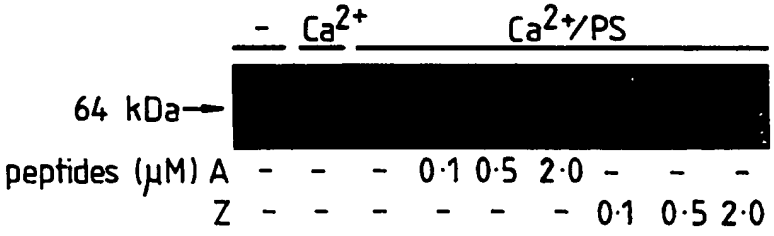


FIG.4

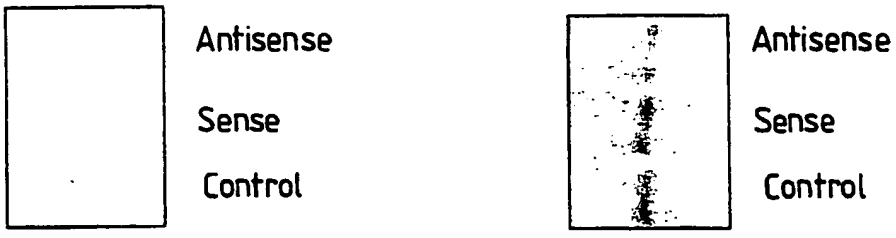


FIG. 5A

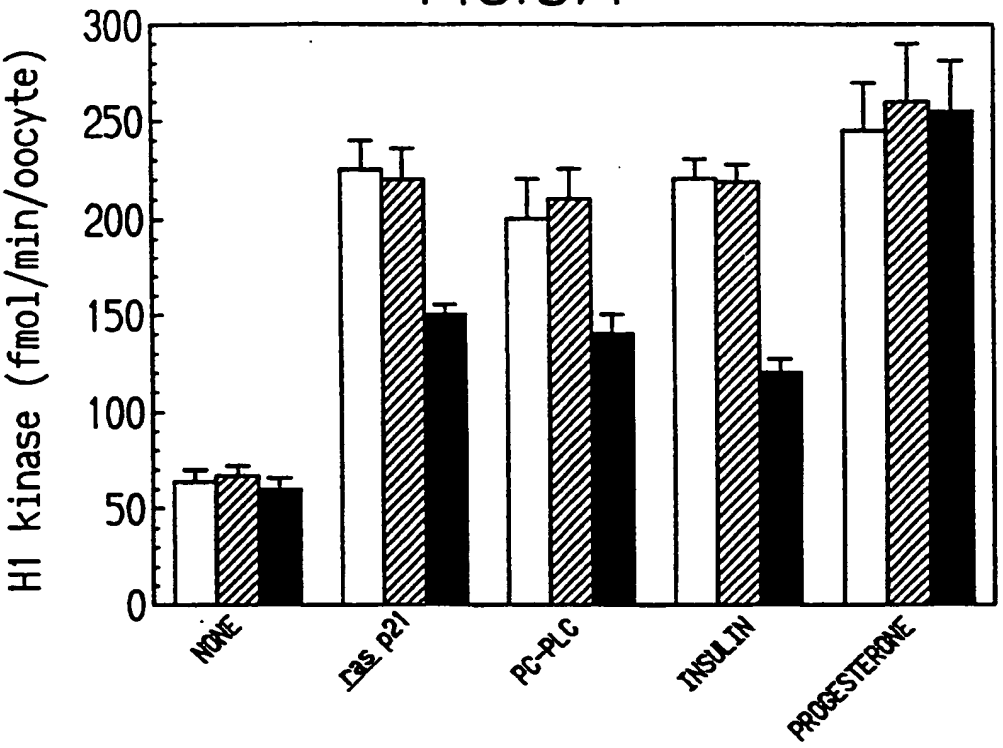

H1 kinase (fmol/min/oocyte)
300
250
200
150
100
50
0
NONE
ras p21
PC-PLC
INSULIN
PROGESTERONE

FIG. 5B

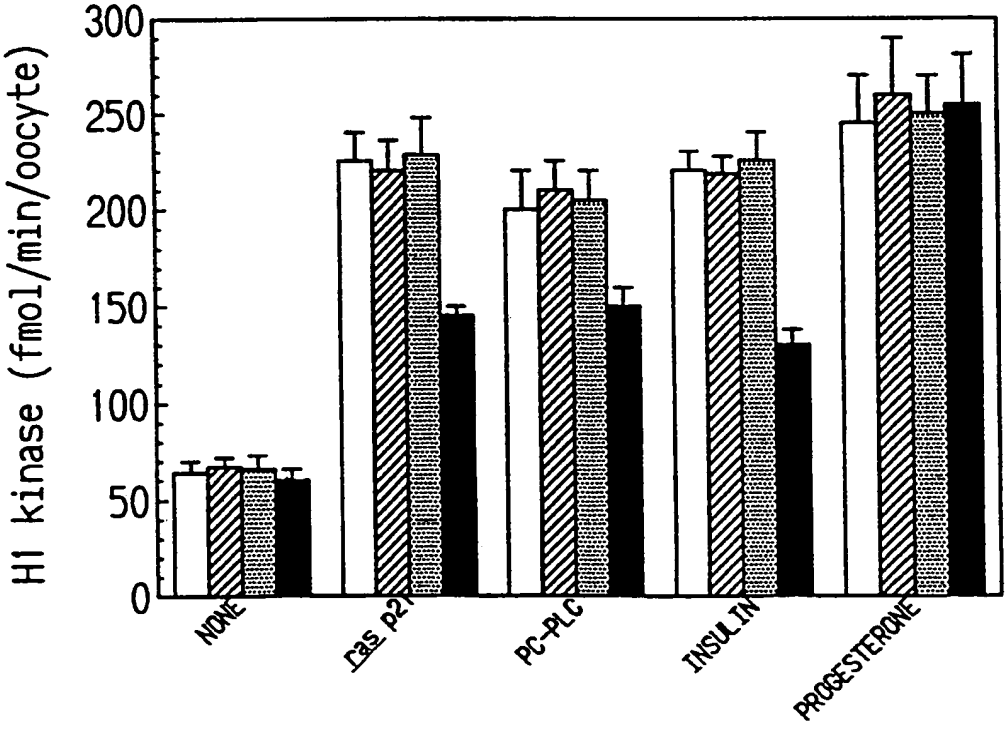

H1 kinase (fmol/min/oocyte)
300
250
200
150
100
50
0
NONE
ras p21
PC-PLC
INSULIN
PROGESTERONE

FIG. 6

PKC	CONSENSUS SEQUENCE	PEPTIDE
α, β, γ	R K G A L R Q K N	SEQ ID NO 6 (A)
ε	R Q G A V R R R V	SEQ ID NO 8 (E)
δ	R R G A I K Q A K	SEQ ID NO 7 (D)
ζ	R R G A R R W R K	SEQ ID NO 5 (Z)
ζ	A R R W R K	SEQ ID NO 4 (Z1)
	A R R W R	SEQ ID NO 3 (Z2)
	A R R W	SEQ ID NO 2 (Z3)
	A R R	SEQ ID NO 1 (Z4)
α, β, γ	A L R	SEQ ID NO 9 (A4)

FIG. 8

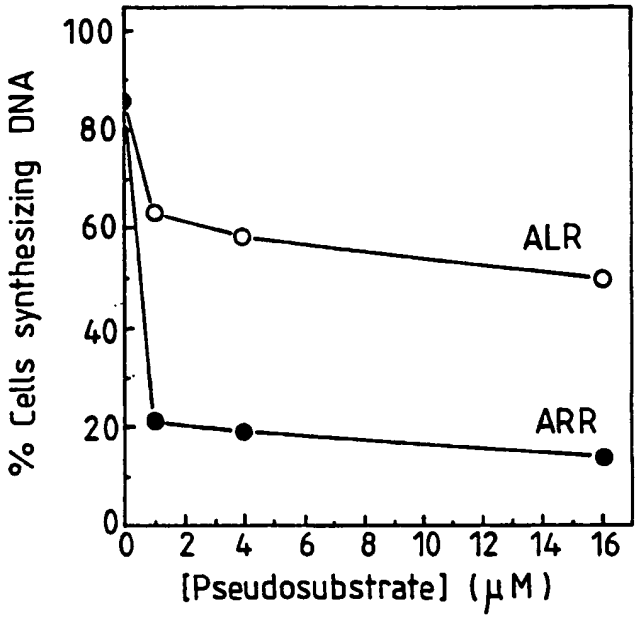
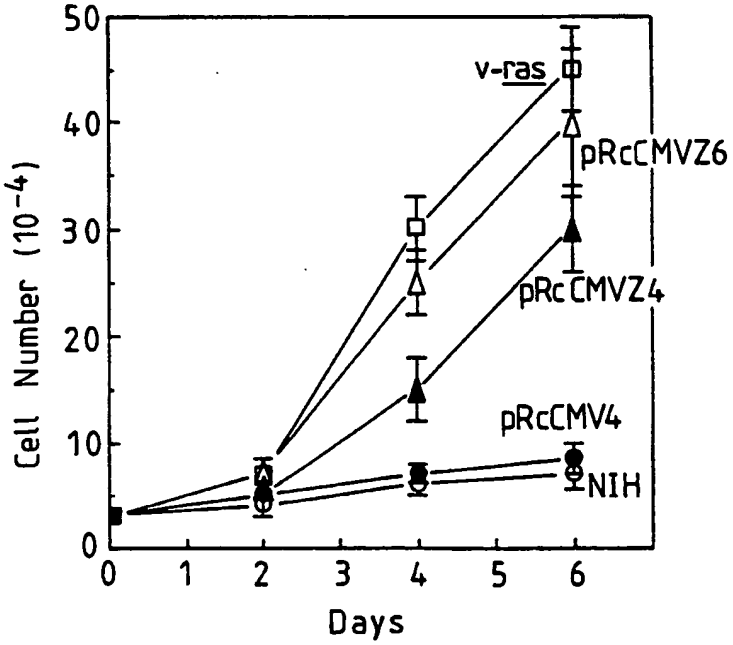

% Cells synthesizing DNA
100
80
60
40
20
0
0 2 4 6 8 10 12 14 16
[Pseudosubstrate] (μM)
ALR
ARR

FIG. 9


Cell Number (10^{-4})
50
40
30
20
10
0
0 1 2 3 4 5 6
Days
v-ras
pRcCMVZ6
pRcCMVZ4
pRcCMV4
NIH

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 93/00816

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C07K5/08; C07K5/10; C12N15/11; C07K7/06
A61K37/64; A61K31/70

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C07K ; C12N ; A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	DD,C,260 084 (KARL-MARX-UNIVERSITÄT) 14 September 1988 see the whole document ---	1-6
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 84, no. 9, May 1987, WASHINGTON US pages 2708 - 2712 D.A.TOWLER 'Purification and characterisation of yeast myristoyl CoA:protein N-myristoyltransferase' see table 2 --- -/--	1,2,6

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
23 JULY 1993	12.08.93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	GROENENDIJK M.S.M.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 86, May 1989, WASHINGTON US pages 3099 - 3103 Y.ONO 'Protein Kinase C zeta subspecies from rat brain: Its structure, expression and properties.' cited in the application	15-18, 21,22,24
X	see figure 2	15
X	CHEMICAL ABSTRACTS, vol. 94, no. 23, 8 June 1981, Columbus, Ohio, US; abstract no. 192678, B.CALAS ET AL 'Field desorption mass spectrometry of basic peptides. An approach in the sequencing of arginine containing peptides' page 699 ;column 2 ; see abstract & BIOMED.MASS SPECTROM. vol. 7, no. 7, 1980, page 288-293	1,2,6,19
Y	SCIENCE vol. 238, 18 December 1987, LANCASTER, PA US pages 1726 - 1728 C.HOUSE E.A. 'Protein kinase C contains a pseudosubstrate prototrope in its regulatory domain' cited in the application see the whole document	1-14,19, 20,22,23
Y	JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 265, no. 36, 25 December 1990, BALTIMORE US pages 22434 - 22440 S.OSADA 'A phorbol ester receptor/protein kinase, nPKC, a new member of the protein kinase C family predominantly expressed in lung and skin' cited in the application see figure 2	1-14,19, 20,22,23

-/--

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 85, February 1988, WASHINGTON US pages 1302 - 1306 K.SUMIKAWA E.A. 'Repression of nicotinic acetylcholine receptor expression by antisense RNAs and an oligonucleotide' cited in the application see the whole document	15-18, 21,22,24
A	JOURNAL OF BIOLOGICAL CHEMISTRY. vol. 266, no. 11, 15 April 1991, BALTIMORE US pages 6825 - 6829 A.G. DE HERREROS E.A. 'Requirement of phospholipase C-catalyzed hydrolysis of phophatidylcholine for maturation of Xenopis laevis oocytes in response to insulin and ras p21' cited in the application see the whole document	1-24
P,X	MOL.CELL.BIOL. vol. 12, no. 9, September 1992, pages 3776 - 3783 I.DOMINGUEZ ET AL 'Evidence for a role of PKC-zeta subspecies in maturation of Xenopus laevis oocytes.' see the whole document	1-24

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 93/00816

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 7, 8 as far as in vivo treatmment concerned and 20-24 are directed to a method of treatment of the human7animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

EP 9300816
SA 72320

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 23/07/93

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DD-C-260084		None	